COMPOSITIONS AND METHODS FOR REGULATED PROTEIN EXPRESSION IN GUT

TECHNICAL FIELD

5   This invention relates to regulatable production of proteins in the gut, and more particularly to nutrient regulated production of glucose-lowering factors from gut endocrine cells.

BACKGROUND

Peptides and proteins, by virtue of their conformational versatility and functional specificity, have been used in treating a host of diseases including diabetes, hemophilia,

10   cancer, cardiovascular disorders, infectious diseases and arthritis (Russell C.S. & Clarke L.A. *Clin Gent* **55**(6):389 (1999); Ryffel B. *Biomed environ Sci* **10**:65(1997); Koths K. *Curr Opin Biotechnol* **6**:681 (1995); Buckel P. *Trends Pharmacol Sci* **17**:450 (1996)). Presently, more than two thirds of the approved biotech medicines are systemic protein drugs. With recent advances in the field of functional genomics, proteomics and genetic

15   engineering, an increasing number of protein drugs are entering the biopharmaceutical market.

Originally, protein drugs were purified from animal tissues or human serum. Protein-based pharmaceuticals have gone through several stages of improvement to reach the current state of clinical application. For example, the biopharmaceutical industry now

20   uses genetically engineered yeast and bacteria to manufacture recombinant human proteins (Scopes R.K. *Biotechnol Appl Biochem* **23**:197 (1996)). This groundbreaking technology has overcome the health risk and shortages that plagued the first generation of protein drugs, and has consequently improved the therapeutic value of proteins. However, despite these advances, broad usage of proteins as therapeutics is still hampered by difficulties in

25   purifying recombinant proteins in active forms and the high cost of manufacturing procedures (Berthold W. & Walter J. *Biologicals* **22**:135(1994); Scopes R.K. *Biotechnol Appl Biochem* **23**:197 (1996)). Additionally, protein drugs face barriers to their entry into the body. When taken orally, they are susceptible to break down by enzymes in the

gastrointestinal tract (Wang W. *J Drug Target* 4:195 (1996); Woodley J.F. *Crit Rev Ther Drug Carrier Syst* 11:61 (1994)).

Other routes of protein delivery explored include infusion pumps (Bremer *et. al., Pharm Biotechnol* 10:239 (1997)) transdermal delivery (Burkoth T.L. *Crit Rev Ther Drug Carrier Syst* 16:331 (1999)), microencapsulation (Cleland J.L. *Pharma Biotechnol* 10:1 (1997)) and inhalation (Gonda I. *J Pharm Sci* 89:940 (2000)). Currently, subcutaneous and intravenous administration by needle injection is the route of choice for delivering protein therapeutics. Unfortunately, this mode of delivery is less than ideal because protein concentrations often are not maintained within a therapeutic range or provide appropriate delivery kinetics. Furthermore, effective treatment with protein drugs usually requires frequent needle injections that can cause local reactions and discomfort, hence resulting in poor patient compliance (Jorgensen J.T. *J Pediatr Endocrinol* 7:175(1994)). These and other factors limit the therapeutic application of many drugs and ultimately hinder their commercial potential. Therefore, it is axiomatic to identify new delivery methods for protein therapeutics.

Insertion of genes encoding specific therapeutic proteins into cells of the body has been used to solve the aforementioned delivery problems in treating diseases. This methodology is referred to as gene therapy and it promises to be the new direction in protein delivery. By this approach, cells in the body can be transformed into 'bioreactors', manufacturing sufficient quantities of therapeutic proteins and hence eliminating the need for frequent needle injections. Currently, gene therapy can be categorized into two general approaches (Drew J. & Martin L-A. In: Lemoine N.R. (ed) *Understanding Gene Therapy*, Springer-Verlag, New York, Chp. 1; pp 1-10 (1999)).

In the first approach, referred to as *in vivo* gene therapy, a gene is introduced in a form that allows its absorption by cells located within the living host. For example, a therapeutic gene is packaged into the genome of viruses such as retrovirus, adeno-associated virus or adenovirus. The recombinant virus containing the therapeutic gene is then introduced into a living organism and allowed to infect cells within the organism. Through the infection process, the virus incorporates its genome containing the therapeutic

genes into the genomic structure of the host cell. As a result, the infected cell expresses the therapeutic gene.

The second approach involves *in vitro* transfer of genetic material to cells removed from the host organism. Following successful incorporation of a gene into the cell's genome, the transformed cells are implanted back into the host. This gene transfer method is referred to as *ex vivo* gene therapy.

Both *in vivo* and *ex vivo* gene therapy offer physicians the power to add or modify specific genes resulting in disease cure (Friedmann T. In: Friedmann T (ed) The Development of Human Gene Therapy. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. Chp 1:pp1-20 (1999)). Clinical applications of this technology are being studied in a wide range of diseases, including cancer, cardiovascular disorders, metabolic diseases, neurodegenerative disorders, immune disorders and other genetic or acquired diseases ((Friedmann T. In: Friedmann T (ed) The Development of Human Gene Therapy. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. Chp 1:pp1-20 (1999); Drew J. & Martin L.-A. In: Lemoine N.R. (ed) *Understanding Gene Therapy*. Springer-Verlag, New York, Chp. 1: pp 1-10 (1999)). Sustained therapeutic concentrations of numerous proteins have been achieved after stable introduction of genes that encode the proteins into cells by gene therapy methodologies. However, for some disorders, regulated delivery of the therapeutic protein is required. For example, insulin replacement therapy for diabetic patients ideally requires that the appropriate amount of insulin be delivered during meals. Likewise, optimal effectiveness of appetite suppressants may be achieved via meal-dependent release. Therefore, to deliver such therapeutic proteins, a release system triggered by a signal or stimuli, such as a meal, is optimal.

A particular disease well suited for timed delivery is diabetes mellitus, a debilitating metabolic disease caused by absent (type 1) or insufficient (type 2) insulin production from pancreatic β-cells (Unger, R.H. et al., *Williams Textbook of Endocrinology* Saunders, Philadelphia (1998)). β-cells are specialized endocrine cells that manufacture and store insulin for release following a meal (Rhodes, *et. al. J. Cell Biol.* 105:145(1987)) and insulin is a hormone that facilitates the transfer of glucose from the

blood into tissues where it is needed. Patients with diabetes must frequently monitor blood glucose levels and many require multiple daily insulin injections to survive. However, such patients rarely attain ideal glucose levels by insulin injection (Turner, R.C. et al. JAMA **281**:2005(1999)). Furthermore, prolonged elevation of insulin levels can

5   result in detrimental side effects such as hypoglycemic shock and desensitization of the body's response to insulin. Consequently, diabetic patients still develop long-term complications, such as cardiovascular diseases, kidney disease, blindness, nerve damage and wound healing disorders (UK Prospective Diabetes Study (UKPDS) Group, *Lancet* **352**, 837 (1998)).

10   Gene therapy represents a promising means to achieve physiologic delivery of therapeutic peptides such as insulin for the treatment of diabetes (Leibowitz, G. & Levine, F. *Diabetes Rev.* 7:124 (1999)). Surrogate cells that express the incorporated gene, process and store the encoded protein, and secrete insulin in regulated fashion therefore affords a treatment for diabetes. Controlling plasma insulin levels by coupling insulin

15   production to changing nutrient requirements of the body also reduces the side effects associated with insulin injection. Accordingly, there is a need for controlled release of proteins to achieve effective treatment of diabetes and other diseases in humans. The present invention satisfies this need and provides related advantages.

SUMMARY

20   The present invention is based, in part, on the production of transformed gut cells that produce insulin in response to glucose. Transformed glucose-responsive cells present in the gut of animals are able to secrete insulin at physiological levels that restore normal glucose homeostasis in diabetic animals. Thus, gut endocrine cells are suitable targets for therapeutic introduction of nucleic acid encoding proteins, *ex vivo* or *in vivo*, whose

25   production in an animal in response to a signal or stimuli (*e.g.*, a nutrient) provides a therapeutic benefit.

The invention therefore provides methods of generating a mucosal cell that produces a protein in response to a nutrient, and compositions including a mucosal cell that produces a protein in response to a nutrient. In one embodiment, a method includes

contacting a mucosal cell with a polynucleotide comprising an expression control element in operable linkage with a nucleic acid encoding a protein under conditions allowing transformation of the cell; and identifying a cell transformant that produces the protein in a nutrient-regulatable manner, thereby generating a mucosal cell that produces a protein in

5   response to a nutrient. In another embodiment, a composition includes an isolated or cultured mucosal cell that produces a protein regulatable by a nutrient, wherein expression of the protein is conferred by a transgene comprising an expression control element in operable linkage with a nucleic acid encoding the protein.

The invention therefore also provides methods of treating a subject having or at

10   risk of having a disorder treatable by producing a protein in a tissue. In one embodiment, a method includes implanting one or more mucosal cells that produce a protein in response to a nutrient into the tissue in an amount effective for treating the disorder. Exemplary implantable tissues include mucosal (e.g., gastrointestinal tract) and non-mucosal (e.g., liver, pancreas or muscle) tissues.

15   Mucosal cells included in the invention include cells that respond to nutrient, which increases expression (e.g., via a nutrient-regulatable expression control element) or secretion of the protein (e.g., secrete a synthesized protein in response to a signal or stimuli, i.e., a "secretagogue").

Nutrients included are natural and non-natural ingestible compounds, such as a

20   sugar, fat, carbohydrate or starch, an amino acid or polypeptide, a triglyceride, a vitamin, a mineral, or cellulose. Nutrient-regulatable elements include a gut endocrine promoter, such as a glucose-dependent insulinotropic polypeptide (GIP) promoter. Nutrient-regulatable elements include functional variants thereof (e.g., point mutation) or a functional subsequence of a full-length regulatable element (deleted sequence).

25   Expression control elements in operable linkage with a nucleic acid encoding the protein, can further include a vector (e.g., a viral vector).

Mucosal cells included in the invention are obtained from a subject, such as a mammal (e.g., human), are obtained from a tissue or organ of the gastrointestinal tract or are derived from a cultured cell line of gut origin. Exemplary tissues where mucosal cells

30   can be obtained include the gastrointestinal tract, large or small intestine (jejunum,

duodenum), stomach, esophagus, buccal or mouth tissue. Mucosal cells also include those that can or are adapted for growth in mucosum, even for short periods of time. Mucosal cells include endocrine and non-endocrine cells, K-cells, stem cells, L-cells, S-cells, G-cells, D-cells, I-cells, Mo-cells, Gr-cells and entero-endocrine cells.

5   Invention compositions and methods include therapeutic proteins such as insulin, leptin, GLP-1, GLP-2, cholecystokinin, a glucagon antagonist, Ghrelin, growth hormones, clotting factors, or antibodies.

The invention therefore also provides methods of treating a subject having, or at risk of having, a disorder treatable by producing a therapeutic protein in a mucosal tissue.

10   In one embodiment, a method includes contacting mucosal cells in the subject that have been transformed with a polynucleotide, for example, an expression control element in operable linkage with a nucleic acid encoding the therapeutic protein, with a nutrient that induces production of the protein in an amount effective to treat the disorder.

Conditions and disorders treatable with the invention methods and compositions

15   include hyperglycemic conditions, such as insulin-dependent and -independent diabetes or where fasting plasma glucose levels are greater than 110 mg/dl; obesity or an undesirable body mass.

The invention therefore also provides animal and genetic models. In one embodiment, a non-human transgenic animal that produces a therapeutic protein (e.g.,

20   insulin) in a mucosal tissue is provided. In one aspect, therapeutic protein production does not naturally occurr in the mucosal tissue of the animal, is conferred by a transgene present in the mucosal tissue, and the transgene includes a polynucleotide including an expression control element in operable linkage with a nucleic acid encoding the protein, wherein production of the protein in the mucosal tissue of the animal is responsive to the

25   nutrient. In one aspect, the protein comprises insulin. The transgenic animal can therefore be made resistant to developing a hyperglycemic condition. A transgenic animal having or at risk of having a hyperglycemic condition can therefore be made to have less glucose or less likely to develop hyperglycemia. In another aspect, the animal is a mouse (e.g., diabetic or hyperglycemic or obese mouse). In yet another aspect, the expression control

30   element conferring expression comprises a nutrient-regulatable element, a functional

variant thereof, or a functional subsequence thereof. In still another aspect, the expression control element includes a glucose-inducible promoter, for example, a glucose-dependent insulinotropic polypeptide (GIP) promoter. Expression of the protein in the animal can be conferred in gastrointestinal tract, intestine/gut, stomach. Cells or tissues of the transgenic

5 animal that produce insulin in response to the nutrient can be isolated. Cells that express protein and also can be isolated include K cells, stem cells and endocrine or non-endocrine cells.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and

10 advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 shows visualization of green fluorescence protein (GFP) expression driven by the GIP promoter in tumor-derived intestinal endocrine cells, STC-1. The left panel

15 shows a sample bright-field population of cells. The same field is seen in the right panel under fluorescence allowing identification of GFP-expressing cells. Fluorescent cell clusters were selected and expanded in culture to generate the K cell line, GTC-1.

FIG. 2 is a representative Northern blot analysis of GIP mRNA in STC-1 and GTC-1 cells showing that GTC-1 is a highly enriched population of GIP-producing K

20 cells.

FIG. 3 is a schematic view of the GIP Ins plasmid construct used for targeting human insulin expression to K cells. It contains the genomic sequence of the human insulin gene operably linked to the GIP promoter (2.5 kb of 5'-regulatory sequence of the GIP gene The three exons are denoted by filled boxes (E1, 2 and 3). The positions of

25 primers used for RT-PCR detection of proinsulin mRNA are indicated. Hind III (H), Pvu II (P) and Xho I (X) sites are shown. Positions of start (ATG) and stop codons are indicated.

FIG. 4 shows expression of human insulin and proinsulin processing enzymes in tumor-derived K cells. The upper panel shows RT-PCR analysis of cDNA from human

islets (H) and GTC-1 cells either transfected (T) or untransfected (UT) with GIP/Ins

plasmid. Samples were prepared with (+) or without (-) reverse transcriptase. The lower

panel shows immunoblot analysis of proprotein convertases PC1/3 and PC2 expression in

GTC-1 cells and a β-cell line (INS-1). Arrows indicate predicted product size for PC1/3

5   isoforms (64 and 82 kD) and PC2 isoforms (66 and 75 kD).

FIG. 5 shows the levels of human insulin and C-peptide detected in culture media

from GTC-1 cells transfected (T) or untransfected (UT) with the GIP/Ins construct.

Insulin and C-peptide are indicated by open and solid bars respectively.

FIG. 6 is a graph showing the stimulatory effect of glucose on insulin secretion

10   from GTC-1 cells stably transfected with the GIP/Ins plasmid.

FIG. 7 shows co-expression of glucokinase (GK, red) and GIP (green) in mouse

duodenal sections.

FIG. 8 shows genomic Southern blot and PCR identification of transgenic founder

lines. Mouse numbers are indicated at the top.

15   FIG. 9 shows targeted expression of human insulin to K cells in transgenic mice

harboring the GIP/Ins construct . The upper panel shows a representative Northern blot

analysis for human insulin gene expression in human islets, control duodenum (mouse)

and transgenic mouse tissues. The lower panel shows RT-PCR analysis of cDNA from

human islets (H), mouse islets (M) and duodenum samples (D) from two transgenic mice

20   using human or mouse specific proinsulin primers. Samples were prepared in the presence

(+) or absence (-) of reverse transcriptase. O indicates no DNA and M indicates markers.

FIG. 10 shows the results of immunohistochemical staining for human insulin in

sections of stomach (left panel) and duodenum (middle panel) from a transgenic mouse.

Arrows indicate human insulin immunoreactive cells. The right panel shows duodenal

25   sections from the same animal examined by immunofluorescence microscopy following

co-staining with antisera specific for insulin (INS, green) and GIP (red).

FIG. 11A is a graph showing that the production of human insulin from gut K cells

of transgenic mice is meal-regulated.

FIG. 11B is a graph showing the release kinetics of human insulin from gut K cells

30   of transgenic mice in response to a mixed meal test and oral glucose challenge.

FIG. 12 is a graph showing the changes of blood glucose concentration in normal control mice, streptozotocin (STZ)-treated control mice and STZ-treated transgenic mice following an oral glucose challenge (1.5 g glucose / kg body weight).

FIG. 13 is a series of micrographs showing immunohistochemical staining for mouse insulin in pancreatic sections from control and STZ-treated transgenic mice. Arrows indicate islets.

FIG. 14 are nucleotide sequences of rat GIP and mouse chromagranin A gene promoter regions.

FIG. 15 are nucleotide sequences of promoter and exon 1 of mouse secretogranin II (Accession no. AF037451) and a 5' portion of mouse glucokinase gene promoter (Accession no. U93275).

FIG. 16 are nucleotide sequences of a 3' portion of mouse glucokinase gene promoter (Accession no. U93275), human adenosine deaminase gene promoter region (Accession no. X02189); and human pre-proinsulin amino adic sequence, and 60 bp of a 5' region of pre-proinsulin.

FIG. 17 are nucleotide sequences of the remaining 3' portion of human pre-proinsulin and a 5' portion of the human leptin gene cDNA.

FIG. 18 are nucleotide sequences of the remaining 3' portion of human leptin, human CCK amino acid and nucleotide sequences and 60 bp of rat CCK promoter.

FIG. 19 are nucleotide sequences of the remaining 3' portion of rat CCK promoter and amino acid and nucleotide sequences of human growth hormone.

FIG. 20 is the sequence for the rat GIP promoter from 1 to - 1894 bp.

DETAILED DESCRIPTION

The invention is based, in part, on the targeted production of a protein in a tissue of animals at levels sufficient to provide therapy. More specifically, the invention includes methods of targeting expression of any protein of interest to endocrine cells in the gastrointestinal tract of a subject such that the protein is released into the bloodstream of the subject in a regulated manner. Genetic constructs including an expression control element (e.g., promoter) that targets gene expression to gut endocrine cells operably linked

to nucleic acid encoding a therapeutic protein can be used. When the gene construct is incorporated into the endocrine cells, the encoded protein will be expressed and secreted in a regulated manner. The transformed endocrine cells expressing the protein encoded by the nucleic acid of interest can secrete a therapeutically effective amount of the protein

5 into the bloodstream of the subject upon feeding of a substance (e.g., nutrient) that increases production of the protein.

Delivery of a genetic construct comprised of a GIP promoter operably linked to a human insulin gene in mice successfully targeted expression and secretion of human insulin by K cells in the gastrointestinal tract of transgenic offspring. Furthermore, the

10 production of human insulin in the transgenic animals was meal regulated. The amount of insulin secreted by the cells was sufficient to protect the transgenic mice from developing diabetes after destruction of pancreatic β-cells. Insulin production was also sufficient to provide normal glucose homeostasis. Thus, introduction of a gene encoding therapeutic proteins such as insulin into meal-regulated endocrine cells in the gut of an animal, either

15 by in vivo or by ex vivo methods (e.g., transplanting in vitro transformed cells that secrete insulin into an animal), can be used to treat disorders treatable by production of a protein.

In accordance with the invention, there are provided methods of generating a mucosal cell that produces a protein regulatable by a nutrient. A method of the invention includes contacting a mucosal cell with a polynucleotide comprising an expression control

20 element in operable linkage with a nucleic acid encoding a protein under conditions allowing transformation, and identifying a transformed cell that produces the protein in a nutrient-regulatable manner. In one embodiment, the mucosal cell is contacted with the polynucleotide in vivo. In another embodiment, the mucosal cell is contacted with the polynucleotide in vitro. In yet another embodiment, the mucosal cell contacted with the

25 polynucleotide in vitro is suitable for transplantation into an animal. In additional embodiments, the mucosal cell is an endocrine cell (e.g., a K cell), or a non-endocrine cell. In still further embodiments, the mucosal cell is a stem cell or a pluripotent or multipotent progenitor cell.

In another embodiment, a nucleic acid expression construct used in the invention is

30 designed to target production of proteins in gastrointestinal endocrine cells. The construct

contains an expression control element operably linked to desired nucleic acid sequences. Expression control elements include promoters capable of targeting expression of a linked nucleic acid of interest to endocrine cells in the gut. Introduction of constructs into target cells can be carried out by conventional methods well known in the art (osmotic shock (*e.g.*, calcium phosphate), electroporation, viral vectors, vesicles or lipid carriers (*e.g.*, lipofection), direct microinjection, etc.).

Typically cell transformation employs a vector. The term "vector," refers to, *e.g.*, a plasmid, virus, such as a viral vector, or other vehicle known in the art that can be manipulated by insertion or incorporation of a polynucleotide, for genetic manipulation (*i.e.*, "cloning vectors"), or can be used to transcribe or translate the inserted polynucleotide (*i.e.*, "expression vectors"). Such vectors are useful for introducing polynucleotides, including a nutrient-regulatable expression control element in operable linkage with a nucleic acid, and expressing the transcribed antisense or encoded protein in cells *in vitro* or *in vivo*.

A vector generally contains at least an origin of replication for propagation in a cell. Control elements, including expression control elements (*e.g.*, nutrient-regulatable) as set forth herein, present within a vector, are included to facilitate transcription and translation. The term "control element" is intended to include, at a minimum, one or more components whose presence can influence expression, and can include components other than or in addition to promoters or enhancers, for example, leader sequences and fusion partner sequences, internal ribosome binding sites (IRES) elements for the creation of multigene, or polycistronic, messages, splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA, polyadenylation signal to provide proper polyadenylation of the transcript of a gene of interest, stop codons, among others.

Vectors can include a selection marker. As is known in the art, "selection marker" or equivalents means genes that allow the selection of cells containing the gene. "Positive selection" refers to a process whereby only the cells that contain the positive selection marker will survive upon exposure to the positive selection agent or be marked. For example, drug resistance is a common positive selection marker; cells containing the

positive selection marker will survive in culture medium containing the selection drug, and those which do not contain the resistance gene will die.

Suitable drug resistance genes are *neo*, which confers resistance to G418, or *hygr*, which confers resistance to hygromycin, or *puro* which confers resistance to puromycin, among others. Other positive selection marker genes include genes that allow the sorting or screening of cells. These genes include genes for fluorescent proteins, the lacZ gene, the alkaline phosphatase gene, and surface markers such CD8, among others.

Vectors included in the invention can contain negative selection markers. "Negative selection" refers to a process whereby cells containing a negative selection marker are killed upon exposure to an appropriate negative selection agent which kills cells containing the negative selection marker. For example, cells which contain the herpes simplex virus-thymidine kinase (*HSV-tk*) gene are sensitive to the drug gancyclovir (GANC). Similarly, the *gpt* gene renders cells sensitive to 6-thioxanthine.

Vectors included in the are those based on viral vectors, such as simian virus 40 (SV40) or bovine papilloma virus (BPV), which has the ability to replicate as extra-chromosomal elements (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982; Sarver *et al.*, *Mol. Cell. Biol.* **1**:486 (1981)). Viral vectors include retroviral, adeno-associated virus, adenovirus, reovirus, lentivirus, rotavirus genomes etc, modified for introducing and directing expression of a polynucleotide or transgene in mucosal cells (Cone *et al.*, *Proc. Natl. Acad. Sci.* USA **81**:6349 (1984)).

"Expression control elements" include polynucleotides, such as promoters and enhancers, that influence expression of an operably linked nucleic acid. Expression control elements and promoters include those active in a particular tissue or cell type, referred to herein as a "tissue-specific expression control elements/promoters." Tissue-specific expression control elements are typically active in specific cell or tissue because they are recognized by transcriptional activator proteins, or other regulators of transcription, that are unique to a specific cell or tissue type.

A particular class of a tissue specific promoter is a "gut endocrine cell specific promoter," a promoter that drives expression of an operably linked nucleic acid in a gut endocrine cell. The GIP promoter is a specific example of a gut endocrine cell promoter.

The GIP promoter includes multiple regulatory sequences which confer specific expression in the gastrointestinal tract (Tseng, C.C. *et al. Proc. Natl. Acad. Sci.* USA **90**:1992 (1993); Yeng, C.M., *et al. Mol. Cell. Endocrinol.* **154**:161 (1999)). A GIP promoter sized about 2.5 Kb (-1 to ~ -2500 bp) in length targeted transgene expression to

5   the stomach and duodenum (Fig. 9 & 10). A shorter GIP promoter (-1 to ~ -1200 bp) conferred expression of transgene in the stomach but not duodenum and miss-targeted transgene expression to the pancreas (Yeng, C.M., *et al. Mol. Cell. Endocrinol.* **154**:161 (1999)). The regulatory sequence between −1200 to −2500 bp therefore appears necessary for targeting transgene expression to GIP producing cells in the intestine.

10   Characterization of transcriptional elements in the GIP promoter revealed two TATA boxes (-27 to −23 and −115 to -111) and two CCAAT-like boxes (-158 to −154 and −170 to -167), potential AP-1 and AP-2 sites, cAMP response element (CRE), and a potential insulin response element (IRE) upstream of the putative transcription start site. Two putative GATA binding motifs also have been identified in the GIP promoter (-178 to

15   −172 (proximal GATA); CAGATAC and −190 to −184 (distal GATA); CAGATAA) which conform to the consensus GATA binding motif sequence, (A/T)GATA(A/G). Specific mutations in the GIP promoter distal and proximal GATA motifs resulted in approximately 90% and 35% reduction in GIP promoter activity respectively, as assessed by luciferase reporter expression (Boylan *et al., J. Biol. Chem.* **273**:17438 (1997)).

20   However, a GIP promoter with both GATA motifs mutated behaved the same as the promoter with only the distal GATA motif altered. Thus, a GIP promoter containing one or more of the aforementioned nucleotide sequences or variants is an example of a subsequence that can retain glucose-regulatable or tissue specific (gut) expression of an operably linked nucleic acid. Such subsequences and variants can be used to confer

25   glucose-regulatable or cell specific expression of an operably linked nucleic acid *in vitro* or *in vivo*.

An additional example of a tissue-specific control element is the promoter of the proglucagon gene. Similar to the GIP promoter, the proglucagon promoter has multiple control sequences that confer expression in either the gastrointestinal tract or brain and

30   pancreas (Lee, Y.C., *et al. J. Biol. Chem.* **267**:10705 (1992); Gajic and Drucker,

Endocrinol. **132**:1055 (1993). A 1300 bp portion of the upstream rat proglucagon promoter sequence targeted expression of a transgene in the brain and pancreas, but not in the gastrointestinal tract (Efrat S., *et. al. Neuron* **1**:605 (1988)). A longer proglucagon gene promoter (-1 to ~ -2000 bp) directed transgene expression in the intestine, in

5   addition to brain and pancreas (Lee, Y.C., *et al. J. Biol. Chem.* **267**:10705 (1992)). Thus, the portion conferring expression in gut appears to be within a 700 bp region of the promoter located between 1300 and 2000 bp upstream of the proglucagon gene.

Additional tissue-specific expression control elements that may be employed to target the expression of the nucleic acid of interest in gut endocrine cells are listed in

10   Table 1. Many of these promoters are also nutrient-regulatable elements. For example, the GIP promoter includes multiple regulatory sequence which confer expression of an operably linked nucleic acid in response to nutrients.

This list is not intended to be exhaustive of all the possible expression control elements useful for driving gene expression in gut endocrine cells but merely to be

15   exemplary.

Although tissue-specific expression control elements may be active in other tissue, for example, a gut specific expression control element may be active in a non-gut tissue, expression is significantly less than that in the gut tissue, (*e.g.*, for non-gut tissue 6-10 fold less than in a gut tissue). Targeted delivery of a vector to gut tissue can limit the

20   possibility of expression elsewhere in the body (*e.g.*, in non-target tissues). Accordingly, tissue-specific elements included herein need not have absolute tissue specificity of expression.

TABLE 1

Exemplary Promoters and Enhancers for Targeting Expression of Proteins to Endocrine Cells in the Gut

Glucokinase
Chromogranin A and B
Cholecystokinin
Glucose-dependent insulinotropic polypeptide
Proglucagon
Adenosine deaminase
Secretin
Gastrin
Somatostatin
Motilin
Ghrelin

Additional expression control elements can confer expression in a manner that is regulatable, that is, a signal or stimuli increases or decreases expression of the operably linked nucleic acid. A regulatable element that increases expression of the operably linked nucleic acid in response to a signal or stimuli is also referred to as an "inducible element" (*i.e.*, is induced by a signal, *e.g.*, a nutrient). A regulatable element that decreases expression of the operably linked nucleic acid in response to a signal or stimuli is referred to as a "repressible element" (*i.e.*, the signal decreases expression such that when the signal, is removed or absent, expression is increased). Typically, the amount of increase or decrease conferred by such elements is proportional to the amount of signal or stimuli present; the greater the amount of signal or stimuli, the greater the increase or decrease in expression.

A particular example of a regulatable expression control element is an element that increases or decreases expression of an operably linked nucleic acid in response to or withdrawal of a nutrient, in which case the element is referred to as a "nutrient-regulatable element." A nutrient inducible or repressible element generally provides basal levels of transcription (*i.e.*, levels of expression in the absence of a stimuli or signal). Typically, basal levels of transcription are greater for a repressible element than for an inducible element.

As used herein, the term "nutrient" means any ingestible or consumable material such as that present in food or drink. As there are many, perhaps billions of different organic and inorganic substances present in food or drink, the term is used broadly herein. Particular examples of nutrients include sugars (e.g., glucose, lactose, sucrose, fructose,

5   mannose, etc.), carbohydrates, starches, fats (saturated or unsaturated), lipids, fatty acids, triglycerides, polypeptides, amino acids, cellulose, hormones, vitamins, and minerals.

Nutrients may also modulate translation or stability of a protein. "Nutrient-regulatable" therefore includes situations where the nutrient modulates transcription, translation of the transcript into protein, or stability of the protein, thereby increasing or

10   decreasing the amount of transcript or protein.

An expression control element can be "constitutive," such that transcription of the operably linked nucleic acid occurs without the presence of a signal or stimuli. Additionally, expression control elements also include elements that confer expression at a particular stage of the cell cycle or differentiation. Accordingly, the invention further

15   includes expression control elements that confer constitutive, regulatable (e.g., nutrient-regulatable), tissue-specific, cell cycle specific, and differentiation stage specific expression.

Expression control elements include full-length sequences, such as native promoter and enhancer elements, as well as subsequences or polynucleotide variants which retain all

20   or part of full-length or non-variant function (e.g., retain some amount of nutrient regulation or cell-specific expression). As used herein, the term "functional" and grammatical variants thereof, when used in reference to a nucleic acid or polypeptide sequence, subsequence or fragment, or nucleotide or amino acid sequence variant, means that the sequence has one or more functions of native nucleic acid or polypeptide sequence

25   (e.g., non-variant or unmodified sequence). As used herein, the term "variant" means a sequence (nucleotide or amino acid) substitution (e.g., point mutation), deletion (internal or external) or addition (e.g., chimeric polypeptide), or other point mutation modification (e.g., chemical derivatives such as modified forms resistant to proteases or nucleases). Typically, amino acid variants have a few or several amino acid changes (e.g., 1 to 10, 10

30   to 20, 20 to 50) such as one or more conservative amino acid substitutions, or non-

conservative amino acid substitutions outside of domains critical to a functionality that is desired to be retained in the variant (e.g., for insulin, glucose lowering function).

Expression control elements, such as nutrient-regulatable elements, also include functional variants, or subsequences. For example, a subsequence of a glucose-regulatable

5   ~2.5 Kb GIP promoter (e.g., 2 Kb, 1 Kb, 0.5 Kb, 0.25 Kb, 0.20 Kb, 100 bp or less) can retain glucose-regulatable or tissue specific (gut or pancreas or brain) expression of an operably linked nucleic acid. Functional domains of various promoters having known properties can be configured to optimize amounts and patterns of expression of the operably linked nucleic acid.

10   Expression control elements included herein can be from bacteria, yeast, plant, or animal (mammalian or non-mammalian), so long as they function to confer expression control of an operably linked nucleic acid. Thus, any expression control element induced by a substance or stimuli (e.g., nutrient) from any organism can be used to modulate transcription of an operably linked nucleic acid in a mucosal cell and, as appropriate,

15   translation of the encoded protein in response to the substance or stimuli, as set forth herein.

Nutrient-regulatable expression control elements exist, for example, as promoters that regulate expression of enzymes involved in glycolysis, lipid metabolism, carbohydrate metabolism and cholesterol (e.g., steroid) metabolism, which are modulated by sugars,

20   fats, carbohydrate, and cholesterol, respectively, and are applicable in the invention. Particular examples of nutrient-regulatable control elements are glucose inducible elements that drive expression of L-pyruvate kinase, acetyl-CoA-carboxylase, spot-14, fatty acid synthase, glyceraldehyde phosphate dehydrogenase phospho-enol-pyruvate carboxykinase, glucose-6-phosphatase and phosphofructokinase (see, also, e.g., Rutter,

25   GA et al., News Physiol Sci. 15:149 (2000)). Another example of a nutrient-regulatable control element is the alcohol-dehydrogenase gene regulatory element. Yet another example of a nutrient-regulatable control element is the vitamin-D response element, which confers expression in the presence of vitamin D. The mammalian metallothionein gene promoter is an expression control element inducible by metals. As with tissue-

30   specific control elements, nutrient-regulatable control elements may be responsive to

multiple nutrients. For example, a glucose-inducible element may also be responsive to lactose. A particular nutrient (e.g., glucose) is therefore not meant to be exclusive of other nutrients in that other nutrients may modulate activity (increase or decrease), to a lesser degree, of the control element.

5   An example of a bacterial nutrient-regulatable expression control element is the lac repressor, which is inducible by beta-galactosides. An example of a yeast nutrient-regulatable expression control element is the gal promoter present in GAL1 and GAL10 genes, which confer galactose-inducible expression. These elements can be operably linked to a nucleic acid and introduced into a mucosal cell in order to confer nutrient-

10   regulatable production of the encoded protein.

Additional expression control elements included are those that are responsive to non-nutrients. Particular examples are chemicals or drugs that are orally active but not normally found in food. The non-nutrient drug or chemical, when consumed, stimulates expression of a nucleic acid operably linked to the non-nutrient expression control

15   element. Ingesting specific amounts of the chemical or drug provides control of the amount of nucleic acid or protein produced (via transcription or secretion). For example, where a drug inducible expression control element confers expression of a nucleic acid encoding insulin, greater amounts of insulin can be produced in the gut by increasing the amount of drug consumed. Particular examples of such non-nutrient expression control

20   systems can be found, for example, in U.S. Patent Nos. 5,989,910; 5,935,934; 6,015,709; and 6,004,941.

As used herein, the term "operable linkage" or grammatical variations thereof refers to a physical or functional juxtaposition of the components so described as to permit them to function in their intended manner. In the example of an expression control

25   element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid.

Expression control can be effected at the level of transcription, translation, splicing, message stability, etc. Typically, an expression control element that modulates transcription is juxtaposed near the 5' end of the transcribed nucleic acid (i.e.,

30   "upstream"). Expression control elements can also be located at the 3' end of the

transcribed sequence (*i.e.*, "downstream") or within the transcript (*e.g.*, in an intron). Expression control elements can be located at a distance away from the transcribed sequence (*e.g.*, 100 to 500, 500 to 1000, 2000 to 5000, or more nucleotides from the nucleic acid). Expression of the operably linked nucleic acid is at least in part controllable

5 by the element (*e.g.*, promoter) such that the element modulates transcription of the nucleic acid and, as appropriate, translation of the transcript. A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. Another example of an expression control element is an enhancer, which can be located 5', 3' of the transcribed sequence, or within the transcribed sequence.

10 As used herein, the term "produces" or "production," when used in reference to a protein expressed by a mucosal cell or tissue, means either expression or secretion of the protein by a mucosal cell. Thus, where a mucosal cell produces a protein in response to a signal or stimuli, such as a nutrient, expression or secretion of the protein increases over the amount prior to the signal or stimuli. Production of a protein by the mucosal cell or

15 tissue may be due to increased transcription of the nucleic acid, translation of the transcript, stability of the transcript or protein, or secretion of the encoded protein. Typically, secretion of a protein by a cell increased by a signal or stimuli (*i.e.* a secretagogue) stimulates release of a protein already translated in the cell. Proteins whose secretion is regulated are typically stored in secretory vesicles within endocrine cells.

20 Alternatively, transcription or translation of a nucleic acid encoding the protein, and subsequent secretion of the translated protein, may be increased by a signal or stimuli. Thus, in the example of a non-endocrine cell, a signal or stimuli (*e.g.*, nutrient) may stimulate transcription of nucleic acid encoding the protein (*e.g.*, insulin) via a nutrient-inducible expression control element, and the cell will subsequently secrete the encoded

25 protein following its translation. In the example of an endocrine cell, such as a gut endocrine cell (*e.g.*, K-cell, L-cell, etc.), the expression control element used to confer expression of the protein may or may not be regulatable but in either case a signal or stimuli typically will regulate secretion of the protein from the cell. In this case, the signal or stimuli functions as a secretagogue that stimulates or increases secretion of a protein.

30 Therefore, in endocrine cells, whether expression is or is not nutrient regulatable (*e.g.*, a

constitutive promoter), protein production by the cell is nutrient regulatable because secretion of the protein is modulated by the nutrient. Accordingly, "nutrient-regulatable" also refers to nutrient modulating secretion of a protein from a cell.

5 Increased secretion of a protein by an endocrine cell in response to a signal or stimuli provides a more rapid response to the signal or stimuli in comparison to a protein produced by increasing transcription of a nucleic acid encoding the protein and subsequent secretion, as in a non-endocrine cell. In contrast, in a non-endocrine cell, a signal or stimuli such as a nutrient can increase transcription of a nucleic acid encoding a protein, and the translated protein is subsequently secreted by the cell without need for a signal or 10 stimuli (e.g., nutrient). Thus, for a non-endocrine mucosal cell transformed with a nutrient-regulatable transgene, transcription of the transgene will be nutrient inducible, but secretion does not require the nutrient.

The nucleic acid can encode a therapeutic polypeptide, such as insulin, a glucagon antagonist, leptin, GLP-1 or cholecytoskinin. For example, a subsequence of full-length 15 insulin that retains some ability to lower glucose, provide normal glucose homeostasis, or reduce the histopatholgical conditions associated with chronic or acute hyperglycemia *in vivo* is but one example of a functional subsequence that has one or more activities of its full length counterpart. Similarly, a subsequence or variant of leptin or CCK or a growth hormone, clotting factor or antibody that retains all or some of the ability to suppress 20 appetite or induce weight stabilization or weight loss, stimulate growth, decrease clotting time or bleeding episodes, or provide passive protection against a foreign antigen (e.g., *H. pylori*) are additional examples of a functional sequence or variant that can be expressed in mucosal tissue of an animal to provide therapeutic benefit.

Thus, "polypeptides," "proteins" and "peptides" encoded by the "nucleic acids," 25 include full-length native sequences, as with naturally occurring proteins, as well as functional subsequences, modified forms or sequence variants so long as the subsequence, modified form or variant retains some degree of functionality of the native full-length protein.

As used herein, the term "transgene" means a polynucleotide that has been 30 introduced into a cell or organism by artifice. For example, a mucosal cell having a

21

transgene, the transgene has been introduced by genetic manipulation or "transformation" of the cell. A cell or progeny thereof into which the transgene has been introduced is referred to as a "transformed cell" or "transformant." Typically, the transgene is included in progeny of the transformant or becomes a part of the organism that develops from the

5   cell. Transgenes may be inserted into the chromosomal DNA or maintained as a self-replicating plasmid, YAC, minichromosome, or the like.

Transgenes include any gene that is transcribed into an antisense or encodes a polypeptide. Particular polypeptides encoded by transgenes include detectable proteins, such as luciferase, β-galactosidase, green fluorescent protein (for non-invasive *in vivo*

10   detection), chlorampenicolacetyltransferase, or proteins that are detectable (*e.g.*, immunologically detectable). Detectable proteins are useful for assessing efficiency of cell transformation (*e.g.*, in *in vivo* gene transfer), cell implantation success, as measured by cell survival or proliferation, for example (*e.g.*, after implanting the transformed cell into animal mucosa).

15   Therapeutic proteins include insulin, a particular transgene useful to treat a hyperglycemic condition such as diabetes. Insulin is the primary hormonal modulator of glucose metabolism and facilitates transport of glucose from the blood to key metabolic organs such as muscle, liver and fat. As shown in Example III, insulin production in the gut of transgenic mice by an insulin transgene prevents diabetes in the mice. Insulin is

20   produced in amounts sufficient to restore glucose tolerance and the timing of insulin release restores normal glucose homeostasis.

Another example of a transgene encoding a therapeutic protein to treat a hyperglycemic condition is a glucagon antagonist. Glucagon is a peptide hormone produced by α-cells in pancreatic islets and is a major regulator of glucose metabolism

25   (Unger R.H. & Orci L., *N. Eng. J. Med.* **304**:1518(1981); Unger R.H. *Diabetes* **25**:136 (1976)) . As with insulin, blood glucose concentration mediates glucagon secretion. However, in contrast to insulin glucagon is secreted in response to a decrease in blood glucose. Therefore, circulating concentrations of glucagon are highest during periods of fast and lowest during a meal. Glucagon levels increase to curtail insulin from promoting

30   glucose storage and stimulate liver to release glucose into the blood. A specific example

of a glucagon antagonist is [des-His^1, des-Phe^6, Glu^9]glucagon-NH$_2$. In streptozotocin diabetic rats, blood glucose levels were lowered by ~37% within 15 min of an intravenous bolus (0.75 μg/g body weight) of this glucagon antagonist (Van Tine B.A. *et. al. Endocrinology* **137**:3316 (1996)).

5   Another example of a transgene encoding a therapeutic protein to treat a hyperglycemic condition or undesirable body mass (*e.g.*, obesity) is glucagon-like peptide-1 (GLP-1). GLP-1 is a hormone released from L-cells in the intestine during a meal which stimulates pancreatic β-cells to increase insulin secretion. GLP-1 has additional activities which make it an attractive therapeutic agent for treating obesity and diabetes. For

10   example, GLP-1 reduces gastric emptying, suppresses appetite, reduces glucagon concentration, increases β-cell mass, stimulates insulin biosynthesis and secretion in a glucose-dependent fashion, and likely increases tissue sensitivity to insulin (Kieffer T.J., Habener J.F. *Endocrin. Rev.* **20**:876 (2000)). Therefore, regulated release of GLP-1 in the gut to coincide with a meal can provide therapeutic benefit for a hyperglycemic condition

15   or an undesirable body mass.

GLP-1 analogs that are resistant to dipeptidyl peptidate IV (DPP IV) provide longer duration of action and improved therapeutic value. Thus, transgenes encoding GLP-1 analogs with increased duration of action can be tageted to gut using the invention described herein to provide nutrient regulated production of GLP-1 analogs for treating a

20   hyperglycemic condition or an undesirable body weight.

Another example of a transgene encoding a therapeutic protein to treat a hyperglycemic condition is an antagonist to the hormone resistin. Resistin is an adipocyte-derived factor for which expression is elevated in diet-induced and genetic forms of obesity. Neutralization of circulating resistin improves blood glucose and insulin

25   action in obese mice. Conversely, administration of resistin in normal mice impairs glucose tolerance and insulin action (Steppan CM et. al. *Nature* **409**:307 (2001)). Production of a protein that antagonizes the biological effects of resistin in gut can therefore provide an effective therapy for obesity-linked insulin resistance and hyperglycemic conditions.

Yet another example of a transgene encoding a therapeutic protein to treat undesirable body mass (*e.g.*, obesity) or a hyperglycemic condition is leptin. Leptin, although produced primarily by fat cells, is also produced in smaller amounts in a meal-dependent fashion in the stomach. Leptin relays information about fat cell metabolism and body weight to the appetite centers in the brain where it signals reduced food intake (promotes satiety) and increases the body's energy expenditure. A single daily subcutaneous injection of leptin had only a modest effect on weight reduction in humans yet leptin treatment results in profound decreases of fat mass in rodents as well as reduction in blood glucose (Seufert J. *et. al. Proc Natl Acad Sci* USA. **96**:674(1999). Previous studies have shown that leptin is rapidly degraded in the circulation. Thus, delivery from gut in a regulated fashion will likely enhance the clinical benefit of leptin reducing food intake and body mass, as well as blood glucose.

Yet another example of a transgene encoding a therapeutic protein to treat undesirable body weight (e.g. obesity) or a hyperglycemic condition is the C-terminal globular head domain of adipocyte complement-related protein (Acrp30). Acrp30 is a protein produced by differentiated adipocytes. Administration of a proteolytic cleavage product of Acrp30 consisting of the globular head domain to mice leads to significant weight loss (Fruebis J. *et al. Proc. Natl. Acad. Sci USA* **98**:2005 (2001)). Therefore, targeted expression of a transgene encoding the globular domain of Acrp30 to gut can promote weight loss.

Still another example of a transgene encoding a therapeutic protein to treat undesirable body mass (*e.g.*, obesity) is cholecystokinin (CCK). CCK is a gastrointestinal peptide secreted from the intestine in response to particular nutrients in the gut. CCK release is proportional to the quantity of food consumed and is believed to signal the brain to terminate a meal (Schwartz M.W. *et. al. Nature* **404**:661-71(2000)). Consequently, elevated CCK can reduce meal size and promote weight loss or weight stabilization (*i.e.*, prevent or inhibit increases in weight gain). A nutrient-regulated CCK delivery system can therefore provide therapeutic benefit for the purpose of reducing food intake in persons.

Additional examples of transgenes encoding therapeutic proteins include clotting factors, to treat hemophilia and other coagulation/clotting disorders (*e.g.*, Factor VIII, IX or X); growth factors (*e.g.*, growth hormone, insulin-like growth factor-1, platelet-derived growth factor, epidermal growth factor, acidic and basic fibroblast growth factors, transforming growth factor-β, etc.), to treat growth disorders or wasting syndromes; and antibodies (*e.g.*, human or humanized), to provide passive immunization or protection of a subject against foreign antigens or pathogens (*e.g.*, *H. Pylori*), or to provide treatment of cancer, arthritis or cardiovascular disease.

Additional transgenes encoding a therapeutic protein include cytokines, interferons (*e.g.*, interferon (INF), INF-α 2b and 2a, INF-α N1, INF-β 1b, INF-gamma), interleukins (*e.g.*, IL-1 to IL-10), tumor necrosis factor (TNF-α TNF-β), chemokines, granulocyte macrophage colony stimulating factor (GM-CSF), polypeptide hormones, antimicrobial polypeptides (*e.g.*, antibacterial, antifungal, antiviral, and/or antiparasitic polypeptides), enzymes (*e.g.*, adenosine deaminase), gonadotrophins, chemotactins, lipid-binding proteins, filgastim (Neupogen), hemoglobin, erythropoietin, insulinotropin, imiglucerase, sarbramostim, tissue plasminogen activator (tPA), urokinase, streptokinase, neurite growth factor (NGF) phenylalanine ammonia lyase, brain-derived neurite factor (BDNF), neurite growth factor (NGF), phenylalanine ammonia lyase, thrombopoietin (TPO), superoxide dismutase (SOD), adenosine deamidase, catalase calcitonin, endothelian, L-asparaginase pepsin, uricase trypsin, chymotrypsin elastase, carboxypeptidase lactase, sucrase intrinsic factor, calcitonin parathyroid hormone(PTH)-like, hormone, soluble CD4, and antibodies and/or antigen-binding fragments (e.g, FAbs) thereof (*e.g.*, orthoclone OKT-e (anti-CD3), GPIIb/IIa monoclonal antibody).

The transgenes described herein are particular applications of the invention but are not intended to limit it. In this regard, the skilled artisan could readily envision additional transgenes transcribed into therapeutic antisense or encode therapeutic polypeptides.

Target cells include mucosal cells or cells not normally present in the mucosum that can or have been adapted for growth in mucosum. As used herein, the terms "mucosa" or "mucosal," when used in reference to a cell, means a cell that can grow in mucosa. Mucosal cells include, for example, those cells which are normally found in

animal mucosa, such as a cell of the gut (e.g., mouth (tongue and buccal tissue), esophagus, and stomach, small and large intestine, rectum, anus), the respiratory tract, the lungs and nasopharynx and other oral cavities (e.g., vagina). Thus, a mucosal cell refers to the various cell types that normally reside in the aforementioned regions including stem

5   cells or other multipotent or pluripotent cells that differentiate into the various mucosal cell types. Particular examples of mucosal cells include endocrine cells, such as K cells, L-cells, S-cells, G-cells, D-cells, I-cells, Mo-cells, Gr-cells and entero-endocrine cells. Endocrine cells are generally characterized by their ability to secrete a synthesized protein into the blood in response to a signal or stimuli (a "secretagogue"). Non-endocrine

10   mucosal cells include epithelial cells which line the outer surface of most mucosal tissue, mucous cells, villus cells, columnar cells, stromal cells and Paneth cells. Non-endocrine cells are generally not known to secrete a synthesized protein into the blood in response to a signal or stimuli.

The finding that gut K cells can function as surrogate cells for producing

15   appropriately regulated physiologic levels of insulin in animals indicates a mode of therapy for diabetes, freeing subjects from insulin injections and reducing or even eliminating the associated debilitating complications. As there are possibly billions of K cells are present in the human gut (Sandström O., El-Salhy M., *Mech. Ageing Dev.* **108**:39 (1999)), regulated insulin secretion from a fraction of these cells may be sufficient to

20   achieve therapeutic benefit, including ameliorating symptoms and complications associated with diabetes.

The gut is the largest endocrine organ in the body capable of producing vast quantities of proteins and contains rapidly renewing tissue in which the dividing cells are accessible. Target cells, such as K cells and stem cells, are predominantly located in the

25   upper gut which is readily accessible to non-invasive gene therapy techniques. Thus, non-invasive techniques like oral formulations, endoscopic procedures, or a modified feeding tube allow the deployment of vectors that facilitate integration of the transgene into the host genome. Vectors have already been developed that deliver genes to cells of the intestinal tract, including the stem cells (Croyle *et al.*, *Gene Ther.* **5**:645 (1998); S.J.

30   Henning, *Adv. Drug Deliv. Rev.* **17**:341 (1997), U.S. Patent Nos. 5,821,235 and

6,110,456). Many of these vectors have been approved for human studies. Therefore, gut cells, such as K cells, that secrete a protein, such as insulin, leptin, glucagon antagonist, GLP-1, GLP-2, Ghrelin, cholecystokinin, growth hormone, clotting factors, antibody, among others, in a regulatable fashion is a means with which to treat diabetes, obesity,

5   growth deficiency and other disorders treatable by producing a protein in mucosal tissue.

A partial list of several types of gut endocrine cells, proteins secreted by the cells in response to particular nutrients ("secretagogues") and exemplary functions are shown in Table 2. The proteins, endocrine cells and nutrients are all applicable in the invention.

Table 2

PEPTIDE	CELL TYPE	CELL LOCATION	FUNCTION	SECRETAGOGUES
Gastrin	G-cells	Gastric Antrum (stomach)	increase acid secretion	Amino acids
Somatostatin	D-cells	GI Tract	reduce gut peptide release (paracrine inhibitor)	Intra-luminal acid, free fatty acids, hormones
Glucose-dependent Insulinotropic Polypeptide	K cells	Upper small intestine	Pancreatic bicarbonate secretion, reduce gastric acid release	Acid, bile salts, fatty acids
Glucagon-like peptide-1	L-cells	Lower small intestine	Increase insulin secretion, decrease gastric acid release	Glucose, fat
Glucagon-like peptide-2	L-cells	Lower small intestine	increase mucosal proliferation	Glucose, fat
Cholecystokinin	I-cells	Upper small	increase gall bladder contraction & pancreatic enzyme secretion	Amino acids, fatty acids
Motilin	Mo-cells	Upper small intestine	Increase Gastric motility	Cyclic release, meals
Ghrelin	Gr-cells	Stomach and intestine	orexigenic	yet to be elucidated

As used herein, the term "cultured," when used in reference to a cell, means that the cell is grown *in vitro*. A particular example of such a cell is a cell isolated from a

5   subject, and grown or adapted for growth in tissue culture. Another example is a cell genetically manipulated *in vitro*, and transplanted back into the same or a different subject.

The term "isolated," when used in reference to a cell, means a cell that is separated from its naturally occurring *in vivo* environment. An example of an isolated cell would be a mucosal cell obtained from a subject such as a human. "Cultured" and "isolated" cells may be manipulated by the hand of man, such as genetically transformed. These terms

5   include any progeny of the cells, including progeny cells that may not be identical to the parental cell due to mutations that occur during cell division. The terms do not include an entire human being.

The target mucosal cell may be present in a mucosal tissue or organ of a subject, such as that of the gut (*e.g.*, intestine). Thus, one way in which to introduce the protein in

10   the subjects' mucosum to achieve therapy is to intracellularly deliver a polynucleotide, including an expression control element, in operable linkage with a nucleic acid encoding the protein into cells present in the mucosum of the subject. Alternatively, the mucosal cell can be isolated from an appropriate tissue of a subject, transfected with the transgene and introduced (transplanted) into a tissue (mucosal or other) of a subject. Thus, another

15   way in which to introduce the protein into the subject to achieve therapy is to transfect a polynucleotide, including an expression control element, in operable linkage with a   · nucleic acid encoding the protein into cultured mucosal cells, followed by implanting the transformed cells or progeny into the subject.

Mucosal cells transfected with a transgene include endocrine and non-endocrine

20   cell lines that grow in-culture. For example, a transformed cell of gut origin or lineage, such as an STC-1 or GTC-1 cell, can be implanted into a tissue of a subject. Mucosal cells transfected with a transgene, *in vitro*, *ex vivo* or *in vivo* include endocrine cells (*e.g.*, K-cell, L-cell, G-cell, D-cell, S-cell, I-cell or Mo-cell, Gr-cell) and non-endocrine epithelial, columnar, stromal, villus, Panth, stem cells or other cell types typically present in mucosal

25   tissue of an animal.

The target cell may also be a non-mucosal cell (endocrine or non-endocrine) which can grow or adapted for growth in mucosum or other tissue (even for a limited time, e.g. days or months). For example, a cell may be obtained from a non-mucosal tissue of a subject, transformed with a transgene or polynucleotide, and then transplanted into a tissue

30   of subject (the same or different subject) in order to effect treatment when the transcribed

antisense or encoded protein is produced. Alternatively, a primary cell isolate or an established non-mucosal cell line can be transformed with a transgene or polynucleotide, and then transplanted into a mucosal tissue of a subject.

Thus, to produce an isolated or cultured mucosal cell of the invention, the mucosal

5   cell may be obtained from a tissue or organ of the gastrointestinal tract of a subject, for example. The mucosal cell can then be transfected with the transgene by conventional nucleic acid techniques and propagated. For example, intestinal stem cells can be isolated and then cultured and transfected *in vitro* (Booth, C. *et al.*, *Exp. Cell Res.* **241**:359 (1999); Kawaguchi, A.L. *et al.*, *J. Pediatr. Surg.* **33**:559 (1998)). Cells that contain or express the

10   transgene can be identified using conventional methods, such as Southern, Northern or Western blots, alone or in combination with selection using a selectable marker. Transformed cells can then be re-introduced (transplanted/implanted) into the same or a different tissue of the same or a different subject from which they were originally obtained.

15   If desired, target mucosal endocrine cells may contain multiple transgenes (*i.e.*, two or more). In this way, expression of different proteins encoded by the transgenes can provide an additive or synergistic effect and, in turn, a therapeutic benefit greater than expression of either protein alone. In addition, if the two transgenes are linked to different expression control elements, or secretion of the two encoded polypeptides are regulated by

20   different signals or stimuli (*e.g.*, two different nutrients), the proteins can be produced either independently of each other or in combination (when both of the different nutrients are provided). For example, two transgenes, one encoding GLP-1 and the other encoding insulin, can be constructed in which production is controlled by two different signals, such as glucose and a drug, respectively. Glucose stimulates production of GLP-1 (either by

25   stimulating transcription or secretion, as discussed herein) whereas the drug stimulates production of insulin (either by stimulating transcription or secretion, as discussed herein). Addition of the drug to stimulate production of insulin (again, either by stimulating transcription or secretion) and addition of glucose can stimulate production of GLP-1; increased amounts of drug or glucose could induce even greater amounts of insulin or

30   GLP-1 production. Production of both insulin and GLP-1 by addition of the drug with a

meal (containing glucose) may provide an even greater therapeutic benefit, especially for subjects suffering from severe diabetes, for example. Accordingly, the invention further includes mucosal cells containing multiple transgenes and methods of producing and using them.

5   Thus, in accordance with the invention, there are provided mucosal cell(s) that produces a protein regulatable by a nutrient, where expression of the protein is conferred by a transgene comprising an expression control element in operable linkage with a nucleic acid encoding the protein. In one embodiment, the mucosal cell is an endocrine cell (*e.g.*, a K-cell). In another embodiment, the mucosal cell is a non-endocrine cell. In 10   yet another embodiment, the mucosal cell is a stem cell, or a multipotent or pluripotent progenitor cell. In an additional embodiment, the expression control element confers nutrient-regulatable expression. In one aspect, the nutrient-regulatable element comprises a gut endocrine promoter (*e.g.*, a GIP promoter). In still another embodiment, the nutrient increases secretion of a protein encoded by the nucleic acid. In yet another embodiment, 15   the nucleic acid encodes a therapeutic polypeptide (*e.g.*, insulin, leptin, glucagon-like peptide-1, glucagon-like peptide-2, a glucagon antagonist, cholecystokinin, a growth hormone, a clotting factor, an antibody, among others). In yet another embodiment, the mucosal cell includes two or more transgenes.

The polynucleotides, including an expression control element, in operable linkage 20   with a nucleic acid, can be introduced for stable expression into cells of a whole organism. Such organisms including transgenic animals, are useful for studying the effect of mucosal protein production in a whole animal and therapeutic benefit. For example, as described herein, production of insulin in the gut of a transgenic mouse protects the animal from developing diabetes and from glucose intolerance after destruction of pancreatic β-cells. 25   Mice strains that develop or are susceptible to developing a particular disease (*e.g.*, diabetes, degenerative disorders, cancer, etc.) are also useful for introducing therapeutic proteins as described herein in order to study the effect of therapeutic protein expression in the disease susceptible mouse. Transgenic and genetic animal models that are susceptible to particular disease or physiological conditions are known in the art and are appropriate 30   targets for expressing therapeutic proteins in gut.

Thus, in accordance with the invention, there are provided non-human transgenic animals that produce a protein in mucosal tissue, production not naturally occurring in mucosal tissue of the animal, production conferred by a transgene present in somatic or germ cells of the animal. In one embodiment, the transgene comprises a polynucleotide,

5 including an expression control element in operable linkage with a nucleic acid encoding a therapeutic polypeptide (e.g., insulin, leptin, GLP-1, GLP-2, Ghrelin, CCK, glucagon antagonist, growth hormone, clotting factor, antibody, among others.) In another embodiment, the transgenic animal is a mouse. In yet another embodiment, expression of the therapeutic polypeptide in the mucosal tissue of the animal is responsive to a nutrient.

10 In still another embodiment, secretion of therapeutic polypeptide in mucosal tissue is increased by a nutrient. In a further embodiment, expression of the therapeutic polypeptide in mucosal tissue is increased by a nutrient (i.e., the expression control element controlling expression of insulin comprises a nutrient-inducible element). In an additional embodiment, the nutrient-regulatable element comprises a glucose-inducible

15 promoter (e.g., a glucose-dependent insulinotropic polypeptide promoter). In additional embodiments, the mucosal tissue is a tissue or organ of the gastrointestinal tract (e.g., intestine) or gut, and includes endocrine cells. In a further embodiment, isolated cells of the invention transgenic animals that express the therapeutic polypeptide are provided.

The term "transgenic animal" refers to an animal whose somatic or germ line cells

20 bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus. The term "transgenic" further includes cells or tissues (i.e., "transgenic cell," "transgenic tissue") obtained from a transgenic animal genetically manipulated as described herein. In the present context, a "transgenic animal" does not encompass

25 animals produced by classical crossbreeding or in vitro fertilization, but rather denotes animals in which one or more cells receive a nucleic acid molecule. Invention transgenic animals can be either heterozygous or homozygous with respect to the transgene. Methods for producing transgenic animals, including mice, sheep, pigs and frogs, are well known in the art (see, e.g., U.S. Patent Nos. 5,721,367, 5,695,977, 5,650,298, and

30 5,614,396) and, as such, are additionally included.

In accordance with the invention, there are provided methods of treating a subject having, or at risk of having, a disorder treatable by producing a therapeutic protein in a mucosal tissue. In one embodiment, a method of the invention includes contacting mucosal tissue cells in the subject transformed with a polynucleotide (*in vitro*, *ex vivo* or

5   *in vivo*) comprising an expression control element in operable linkage with a nucleic acid encoding the therapeutic protein with a nutrient that induces production of the protein in an amount effective to treat the disorder. In another embodiment, a method of the invention includes producing a therapeutic protein in a mucosal tissue of the subject by implanting one or more transformed mucosal cells (*in vitro* or *ex vivo*) that produce the

10   protein into the subject's tissue in an amount effective for treating the disorder.

Disorders treatable by a method of the invention include a hyperglycemic condition, such as insulin-dependent (type 1) or -independent (type 2) diabetes, as well as physiological conditions or disorders associated with or that result from the hyperglycemic condition. Thus, hyperglycemic conditions treatable by a method of the invention also

15   include a histopathological change associated with chronic or acute hyperglycemia (*e.g.*, diabetes). Particular examples include degeneration of pancreas (β-cell destruction), kidney tubule calcification, degeneration of liver, eye damage (diabetic retinopathy), diabetic foot, ulcerations in mucosa such as mouth and gums, excess bleeding, delayed blood coagulation or wound healing and increased risk of coronary heart disease, stroke,

20   peripheral vascular disease, dyslipidemia, hypertension and obesity.

Thus, in various methods of the invention, a mucosal cell that produces insulin or a functional subsequence of insulin in response to glucose, is useful for increasing insulin, decreasing glucose, improving glucose tolerance, treating a hyperglycemic condition (*e.g.*, diabetes) or for treating a physiological disorders associated with or resulting from a

25   hyperglycemic condition. Such disorders include, for example, diabetic neuropathy (autonomic), nephropathy (kidney damage), skin infections and other cutaneous disorders, slow or delayed healing of injuries or wounds (*e.g.*, that lead to diabetic carbuncles), eye damage (retinopathy, cataracts) which can lead to blindness, diabetic foot and accelerated periodontitis. Such disorders also include increased risk of developing coronary heart

30   disease, stroke, peripheral vascular disease, dyslipidemia, hypertension and obesity.

As used herein, the term "hyperglycemic" or "hyperglycemia," when used in reference to a condition of a subject, means a transient or chronic abnormally high level of glucose present in the blood of a subject. The condition can be caused by a delay in glucose metabolization or absorption such that the subject exhibits glucose intolerance or a

5 state of elevated glucose not typically found in normal subjects (e.g., in glucose-intolerant subdiabetic subjects at risk of developing diabetes, or in diabetic subjects). Fasting plasma glucose (FPG) levels for normoglycemia are less than about 110 mg/dl, for impaired glucose metabolism, between about 110 and 126 mg/dl, and for diabetics greater than about 126 mg/dl.

10 Disorders treatable by producing a protein in a mucosal tissue also include obesity or an undesirable body mass. Leptin, cholecystokinin and GLP-1 decrease hunger, increase energy expenditure, induce weight loss or provide normal glucose homeostasis. Thus, in various embodiments, a method of the invention for treating obesity or an undesirable body mass, or hyperglycemia, includes contacting mucosal tissue cells having

15 a transgene encoding leptin, cholecystokinin or GLP-1 with a nutrient so as to produce the protein in an amount effective to treat obesity or an undesirable body mass. Disorders treatable also include those typically associated with obesity, for example, abnormally elevated serum/plasma LDL, VLDL, triglycerides, cholesterol, plaque formation leading to narrowing or blockage of blood vessels, increased risk of hypertension, stroke, coronary

20 heart disease, etc.

As used herein, the term "obese" or "obesity" refers to a subject having at least a 30% increase in body mass in comparison to an age and gender matched normal subject. "Undesirable body mass" refers to subjects having 1%-29% greater body mass than a matched normal subject as well as subjects that are normal with respect to body mass but

25 who wish to decrease or prevent an increase in their body mass.

The term "subject" refers to an animal. Typically, the animal is a mammal, however, any animal having mucosal tissue, such as gut, is encompassed by the term. Particular examples of mammals are primates (humans), dogs, cats, horses, cows, pigs, and sheep. Subjects include those having a disorder, e.g., a hyperglycemic disorder, such

30 as diabetes, or subjects that do not have a disorder but may be at risk of developing the

disorder, *e.g.*, subdiabetic subjects having FPG levels between about 110 and 126 mg/dl. Subjects at risk of developing a disorder include, for example, those whose diet may contribute to or be associated with development of diabetes or obesity, as well as those which may have a family history or genetic predisposition towards development of

5   diabetes or obesity. Subjects also include apparently normal subjects, for example, those who wish to lose weight but are not considered to be obese or have greater than normal body mass.

A partial list of therapeutic proteins and target diseases is shown in Table 3.

Table 3

LEAD COMPOUNDS	TARGET DISEASE	FUNCTION	THERAPEUTIC EFFECT
Insulin	Diabetes	Insulin replacement	Improve glucose tolerance Delay/prevent diabetes
Glucagon antagonists	Diabetes	Reduce endogenous glucose production	Improve glucose tolerance
GLP-1	Diabetes Obesity	Stimulate growth of β-cells, improve insulin sensitivity, suppress appetite	Improve glucose tolerance Induce weight loss
Leptin	Obesity Diabetes	Appetite suppression and improvement of insulin sensitivity	Induce weight loss Improve glucose tolerance
CCK	Obesity	Appetite suppression	Induce weight loss
Growth hormone (GH)	GH deficiencies, wasting and anti-aging	GH replacement	Improve growth
Clotting factors	Hemophilia	Clotting factors replacement	Improve clotting time
Therapeutic human monoclonal antibodies	Infections Cancers	Pathogen neutralization or immune modulations	Prevent infections or transplant rejections

10   Treatment generally results in reducing or preventing the severity or symptoms of the condition in the subject, *i.e.*, an improvement in the subject's condition or a "therapeutic effect." Therefore, treatment can reduce the severity or prevent one or more symptoms of the condition or an associated disorder, inhibit progression or worsening of

the condition or an associated disorder, and in some instances, reverse the condition or an associated disorder. Thus, in the case of a hyperglycemic condition, for example, treatment can reduce blood glucose, improve glucose tolerance, provide normal glucose homeostasis, or prevent, improve, or reverse a histopathological change associated with or

5   that results from the hyperglycemic condition.

Improvement of a histopathological change associated with a hyperglycemic condition includes, for example, preventing further or reducing kidney tubule calcification, decreasing or arresting retinopathy or cataracts, decreasing wound or injury healing time, reducing diabetic foot, preventing or reducing accelerated periodontitis, or decreasing the

10 risk of developing coronary heart disease, stroke, peripheral vascular disease, dyslipidemia, hypertension and obesity. Improvement in obesity can include, for example, a reduction of body mass or an improvement in an associated disorder, such as a decrease in cholesterol, LDL or VLDL levels, a decrease in blood pressure, a decrease in intimal thickening of the blood vessel associated with high fat diet, a decrease in resting heart rate,

15 an increase in lung capacity, etc. Improvement in a bleeding disorder, such as hemophilia can induce, for example, decreased clotting time or frequency duration of bleeding episodes.

As used herein, the term "ameliorate" means an improvement in the subject's condition, a reduction in the severity of the condition, or an inhibition of progression or

20 worsening of the condition. In the case of a hyperglycemic condition (e.g., diabetes), for example, an improvement can be a decrease in blood glucose, an increase in insulin, an improvement in glucose tolerance, or glucose homeostasis. An improvement in a hyperglycemic condition also can include improved pancreatic function (e.g., inhibit or prevent β-islet cell destruction), a decrease in a pathology associated with or resulting

25 from the condition, such as an improvement in histopathology of an affected tissue or organ, as set forth herein. In the case of obesity, for example, an improvement can be a decrease in weight gain, a reduction of body mass or an improvement in a conditions associated with obesity, as set forth herein (e.g., reduction of blood glucose, cholesterol, LDL or VLDL levels, a decrease in blood pressure, a decrease in intimal thickening of the

30 blood vessel, etc.). In the case of hemophilia or other blood coagulation clotting bleeding

disorders, an improvement can reduce the frequency or duration of bleeding episodes or hemorrhage. Improvements likewise include chronic disorders associated with blood coagulation/clotting/bleeding associated disorders such as a reduction in neurological problems, crippling tissue and joint damage, for example.

5   The doses or "effective amount" for treating a subject are preferably sufficient to ameliorate one, several or all of the symptoms of the condition, to a measurable or detectable extent, although preventing or inhibiting a progression or worsening of the disorder or condition, or a symptom, is a satisfactory outcome. Thus, in the case of a condition or disorder treatable by producing a protein in a mucosal tissue, the amount of

10   protein produced, or transplanted cell(s) sufficient to ameliorate a condition treatable by a method of the invention will depend on the condition and the desired outcome and can be readily ascertained by the skilled artisan. Appropriate amounts will depend upon the condition treated, the therapeutic effect desired, as well as the individual subject (*e.g.*, the bioavailability within the subject, gender, age, etc.). For example, a partial restoration of

15   normal glucose homeostatsis in a subject can reduce the frequency for insulin injection, even though complete freedom from insulin injection has not resulted.

The effective amount can be ascertained by measuring relevant physiological effects. For example, in the case of diabetes or other hyperglycemic condition, a decrease in blood glucose or an improvement in glucose tolerance test can be used to determine

20   whether the amount of insulin, or cell(s) expressing insulin transplanted into the animal mucosa, is effective to treat the hyperglycemic condition. For example, an amount reducing FPG from 126 mg/dl to 120, 115, 110, or less is an effective amount. In the case of obesity or an undesirable body mass, a decrease in the subjects' mass, a decrease in meal size or caloric content of a meal, increased satiety for a given meal size, and

25   decreases in serum/plasma levels of lipid, cholesterol, fatty acids, LDL or VLDL all can be effective amounts for ameliorating obesity or an undesirable body mass of a subject. In the case of hemophilia, an effective amount is an amount which reduces clotting time or frequency or duration of bleeding episodes in a subject.

The methods of the invention for treating a subject are applicable for prophylaxis

30   to prevent a condition in a subject, such as a hyperglycemic condition or an associated

disorder, or development of obesity or an increased body mass. Alternatively, the methods can be practiced following treatment of a subject as described herein. For example, following treatment and a reduction of body mass to the desired weight, leptin, GLP-1 or CCK can be periodically produced by mucosal cells, as described herein, in

5   order to suppress appetite, decrease meal consumption, etc. thereby maintaining desired body weight.

The methods of the invention for treating a subject also can be supplemented with other forms of therapy. Supplementary therapies include drug treatment, a change in diet (low sugar, fats, etc.) surgical resection, transplantation, radiotherapy, etc. For example, a

10   method of the invention for treating a hyperglycemic condition can be used in combination with drugs or other pharmaceutical formulations that increase insulin or lower glucose in a subject. Drugs for treating diabetes include, for example, biguanides and sulphonylureas (e.g., tolbutamide, chlorpropamide, acetohexamide, tolazamide, glibenclamide and glipizide). Appetite suppression drugs are also well known and can be used in

15   combination with the methods of the invention. Supplementary therapies can be administered prior to, contemporaneously with or following the invention methods of treatment. The skilled artisan can readily ascertain therapies that may be used in a regimen in combination with the treatment methods of the invention.

As a method of the invention can include *in vivo* delivery, such as a polynucleotide

20   comprising an expression control element in operable linkage with a nucleic acid into mucosal cells of a subject, in order to produce an encoded protein in the subject, for example, expression systems further include vectors specifically designed for *in vivo* delivery. Vectors that efficiently deliver genes to cells of the intestinal tract (e.g., stem cells) have been developed and are contemplated for use in delivering the polynucleotides

25   into mucosal cells (see, e.g., U.S. Patent Nos. 5,821,235, 5,786,340 and 6,110,456; Croyle, M.A. *et al.*, *Gene Ther.* **5**:645 (1998); Croyle, M.A. *et al.*, *Pharm. Res.* **15**:1348 (1998); Croyle, M.A. *et al.*, *Hum. Gene Ther.* **9**:561 (1998); Foreman, P.K. *et al.*, *Hum. Gene Ther.* **9**:1313 (1998); Wirtz, S. *et al.*, *Gut* **44**:800 (1999)). Adenoviral and adeno-associated viral vectors suitable for gene therapy are described in U.S. Patent Nos.

30   5,700,470, 5,731,172 and 5,604,090. Additional vectors suitable for gene therapy include

herpes simplex virus vectors (see, *e.g.*, U.S. Patent No. 5,501,979), retroviral vectors (see, *e.g.*, U.S. Patent Nos. 5,624,820, 5,693,508 and 5,674,703; and WO92/05266 and WO92/14829), bovine papilloma virus (BPV) vectors (see, *e.g.*, U.S. Patent No. 5,719,054), CMV-based vectors (see, *e.g.*, U.S. Patent No. 5,561,063) and parvovirus,

5   rotavirus and Norwalk virus vectors. Lentiviral vectors are useful for infecting dividing as well as non-dividing cells (see, *e.g.*, U.S. Patent No. 6,013,516).

Introduction of nucleic acid and polypeptide *in vitro*, *ex vivo* and *in vivo* can also be accomplished using other techniques. For example, a polynucleotide comprising an expression control element in operable linkage with a nucleic acid encoding a protein can

10   be incorporated into particles or a polymeric substance, such as polyesters, polyamine acids, hydrogel, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate, or lactide/glycolide copolymers, polylactide/glycolide copolymers, or ethylenevinylacetate copolymers. A polynucleotide can be entrapped in microcapsules prepared by coacervation techniques or by interfacial

15   polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules, or poly (methylmethacrolate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule complexes, nano-capsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The use of liposomes for introducing

20   various compositions, including polynucleotides, is known to those skilled in the art (see, *e.g.*, U.S. Patent Nos. 4,844,904, 5,000,959, 4,863,740, and 4,975,282). A carrier comprising a natural polymer, or a derivative or a hydrolysate of a natural polymer, described in WO 94 20078 and U.S. Patent No. 6,096,291, is suitable for mucosal delivery of molecules, such as polypeptides and polynucleotides. Piperazine based amphilic

25   cationic lipids useful for gene therapy also are known (see, *e.g.*, U.S. Patent No. 5,861,397). Cationic lipid systems also are known (see, *e.g.*, U.S. Patent No. 5,459,127). Accordingly, vector (viral and non-viral, *e.g.*, naked DNA) and non-vector means of delivery into mucosal cells or tissue, *in vitro*, *in vivo* and *ex vivo* can be achieved and are contemplated.

As the methods of the invention can include contacting a mucosal cell(s) present in a subject with a polynucleotide, the present invention also provides "pharmaceutically acceptable" or "physiologically acceptable" formulations in which a transgene or therapeutic polypeptide are included. Such formulations can be administered *ex vivo* or *in*

5   *vivo* to a subject in order to practice the treatment methods of the invention, for example.

As used herein, the terms "pharmaceutically acceptable" and "physiologically acceptable" refer to carriers, diluents, excipients and the like that can be administered to a subject, preferably without producing excessive adverse side-effects (*e.g.*, nausea, abdominal pain, headaches, etc.). Such preparations for administration include sterile

10   aqueous or non-aqueous solutions, suspensions, and emulsions.

Pharmaceutical formulations can be made from carriers, diluents, excipients, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with administration to a subject. Such formulations can be contained in a tablet (coated or uncoated), capsule (hard or soft),

15   microbead, emulsion, powder, granule, crystal, suspension, syrup or elixir. Supplementary active compounds and preservatives, among other additives, may also be present, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

A pharmaceutical formulation can be formulated to be compatible with its intended route of administration. Thus, pharmaceutical formulations include carriers, diluents, or

20   excipients suitable for administration by routes including intraperitoneal, intradermal, subcutaneous, oral (*e.g.*, ingestion or inhalation), intravenous, intracavity, intracranial, transdermal (topical), parenteral, *e.g.* transmucosal and rectal.

Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following: a sterile diluent such as water for injection, saline

25   solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such

as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical formulations suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous

5   preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable

10   mixtures thereof. Fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Isotonic agents, for example, sugars, polyalcohols

15   such as manitol, sorbitol, sodium chloride can be included in the composition. Prolonged absorption of injectable formulations can be achieved by including an agent that delays absorption, for example, aluminum monostearate or gelatin.

For oral administration, a composition can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Pharmaceutically

20   compatible binding agents, and/or adjuvant materials can be included in oral formulations. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or

25   Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or flavoring.

Formulations can also include carriers to protect the composition against rapid degradation or elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. For example, a time delay

material such as glyceryl monostearate or glyceryl stearate alone, or in combination with a wax, may be employed.

Additional formulations include biodegradable or biocompatible particles or a polymeric substance such as polyesters, polyamine acids, hydrogel, polyvinyl pyrrolidone,

5   polyanhydrides, polyglycolic acid, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate, or lactide/glycolide copolymers, polylactide glycolide copolymers, or ethylenevinylacetate copolymers in order to control delivery of an administered composition. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained

10   commercially from Alza Corporation and Nova Pharmaceuticals, Inc., for example.

The rate of release of a composition can be controlled by altering the concentration or composition of such macromolecules. For example, the composition can be entrapped in microcapsules prepared by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly

15   (methylmethacrolate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule complexes, nano-capsules, microspheres, microbeads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

20   Additional pharmaceutical formulations appropriate for administration are known in the art and are applicable in the methods and compositions of the invention (see, e.g., Remington's Pharmaceutical Sciences (1990) 18th ed., Mack Publishing Co., Easton, PA; The Merck Index (1996) 12th ed., Merck Publishing Group, Whitehouse, NJ; and Pharmaceutical Principles of Solid Dosage Forms, Technonic Publishing Co., Inc.,

25   Lancaster, Pa., (1993)).

The mucous or endothelial lining of the mucosal tissue may be removed or otherwise prepared prior to administration, for example, using penetrants or other barrier penetration enhancers. Such penetrants appropriate to the barrier to be permeated are generally known in the art, and include, for example, for transmucosal administration,

30   incubation with N-acetyl-cysteine (Nakanishi *et al. Chem Pharm Bull* (Tokyo) **40**:1252

(1992), Meaney and O'Driscoll *Eur J Pharm Sci.* **8**:167 (1999); hydrolysis of intestinal mucins by purified Sigma 1 protein and infectious subviral particles (Bisaillon *et al. J Mol Biol.* **286**:759(1999); desialation (Slomiany *et al. Gen Pharmacol.* **27**:761 (1996); (Hirmo *et al. FEMS Immunol Med Microbiol.* **20**:275 (1998); desulphation by H. pylori

5   glycosulfatase (Slomiany *et al. Am J Gastroenterol.* **87**:1132 (1992); desialation by neuraminidase (Hanski *et al. Cancer Res.* **51**:5342 (1991)); disulphide bond breakage by β-mercaptoethanol (Gwozdzinski *et al. Biochem Int.* **17**:907 (1988); deglycosylation with specific exoglycosidases such as fucosidase, β-galactosidase, N-acetyl-galactosaminidase, β-N-acetyl hexososaminidase, and neuraminidase (Slomiany *et al. Biochem Biophys Res*

10   *Commun.* **142**:783 (1987); acid removal of by 0.4 N HCl (Ruggieri *et al. Urol Res.* **12**:199 (1984), Davis C.P. and Avots-Avotins A.E. Scan Electron Microsc. (Pt 2):825-30 (1982), Parsons *et. al. Am J Pathol.* **93**:423 (1978)), among others. Mucosal administration can also be accomplished through the use of nasal sprays or suppositories. For administration by inhalation, the formulation can be delivered via a pump or an aerosol spray from a

15   dispenser or pressured container that contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

The number of stem cells can be increased by exposure to cytotoxic agents and growth factors. For example, irradiation of the small gut increases the number clonogenic/stem cells (Roberts S.A. *Radiat. Res.* **141**:303 (1995); Cai W.B. *et. al. Intl. J.*

20   *Radiat. Biol.* **71**:145 (1997)). In addition, treatment with GLP-2, epidermal growth factor, TGF-α, insulin-like growth factors, interleukins, among others, have been shown to promote the growth of mucosal cells (Potten C.S. *Int. J. Exp. Path* **78**:219 (1997)). In this way, additional target cells can be produced thereby increasing transformation efficiency and subsequent regulated protein production by transformed cells.

25   Endoscopes, cannulas, intubation tubes, catheters and the like can be used to deliver the formulation to various parts of the gut of a subject. This allows effective delivery and targeting of vectors to particular areas of the gut.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

30   invention belongs. Although methods and materials similar or equivalent to those

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described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein.

All publications, patents and other references cited herein are incorporated by reference in their entirety. In case of conflict, the present specification, including

5 definitions, will control.

As used herein, the singular forms "a", "and," and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a mucosal cell" includes a plurality of such cells and reference to "a polynucleotide comprising an expression control element in operable linkage with a nucleic acid" includes reference to

10 one or more such constructs, and so forth.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the following examples are intended to illustrate but not limit the scope of invention described in the claims.

15 **EXAMPLE I**

This example describes the establishment of a gut endocrine cell line useful for studying regulated insulin production and for targeting insulin expression *in vivo*. This example also describes construction of a human insulin gene expression vector.

A GIP-expressing cell line was established to investigate whether the GIP

20 promoter is effective in targeting insulin gene expression to K cells. This cell line was cloned from the murine intestinal cell line STC-1, a mixed population of gut endocrine cells (Rindi *et. al., Am. J. Pathol.* **136**:1349 (1990)). K cells in the mixed population were visually identified by transfection of a green fluorescent protein expression plasmid driven by ~2.5 Kb of the rat GIP promoter. The rat GIP promoter was obtained from a rat

25 genomic λDASH library (Stratagene) by plaque hybridization with the rat GIP cDNA clone as described previously (Boylan *et. al., J. Biol. Chem.* **273**:17438 (1997)) and subcloned into the promoterless pEGFP-1 plasmid (Clontech). The resulting reporter vector was transfected into STC-1 cells (D. Drucker, University of Toronto) using Lipofectamine (GIBCO). Cells were dispersed with Trypsin EDTA and fluorescent cells

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expressing EGFP were double hand-picked and placed into individual dishes for clonal expansion (FIG. 1).

Following clonal expansion of the transiently fluorescent cells, clones were analyzed for the expression of GIP mRNA by northern blotting. In brief, total RNA from

5   GTC-1 and STC-1 cells was isolated with Trizol (Gibco) according to manufacturer's instructions. Total cell RNA (20 ug) from each sample was electrophoretically separated and transferred to nylon membrane. Hybridization was performed with radiolabeled 660 bp EcoR1 fragment of the rat GIP cDNA that was random-primed with $[\alpha-^{32}P]dCTP$. Following hybridization, membranes were washed and exposed to x-ray film. The level of

10   GIP mRNA in one clone (GIP Tumor Cells; GTC-1) was ~8-fold higher than in the parental heterogeneous STC-1 cells (FIG 2).

In order to determine if GTC-1 cells correctly process human genomic preproinsulin, an insulin expression construct in which the insulin gene linked to the 3' end of the rat GIP promoter (FIG. 3, GIP/Ins) was transfected into these cells.

15   To construct the human insulin GIP expression plasmid, a ~2.5 Kb portion of the rat GIP promoter was inserted into pGLBH as discussed above (Boylan *et al., J. Biol. Chem.* **273**:17438 (1997)). Human insulin cDNA, which comprises ~1.6 Kb of the genomic sequence extending from nucleotides 2127 to 3732 including the native polyadenylation site, was excised from pBR322 (ATCC No. 57399) by digestion with

20   BamHI and ligated into the BglII site of the GIP containing pGLBH construct. The expression construct is shown in FIG 3.

Total RNA was isolated from GIP Ins-transfected and non-transfected cells and human islets (Trizol, GIBCO). Five μg of the RNA isolated was reversed transcribed with oligo-dT primer using superscript II reverse transcriptase (GIBCO). Two μl of the cDNA

25   product was amplified with human preproinsulin gene-specific primers (Primer 1 and 3, FIG 3). The results indicate that human preproinsulin mRNA transcript was correctly processed (FIG. 4, Top).

When the GIP Ins construct was transfected into a β-cell (INS-1), liver (HepG2) and rat fibroblast (3T3-L1) cell line, little human preproinsulin mRNA was detectable.

45

These observations indicate that the GIP promoter is cell specific and is likely to be effective in targeting transgene expression to K cells *in vivo*.

Western blot analysis of GTC-1 cells to determine if processing enzymes for converting proinsulin to mature insulin were present was then performed. In brief, GTC-1 cells were lysed in ice-cold RIPA buffer and supernatants were assayed for total protein content using the Bradford method. Cell lysate protein (50 μg) was fractionated on 10% SDS-PAGE and fractionated proteins were electroblotted onto nitrocellulose membranes and incubated with polyclonal antibodies recognizing PC1 3 and PC2 (Dr. Iris Lindberg, Louisiana State Medical Center). Membranes were washed, incubated with goat anti-rabbit antisera coupled to horseradish peroxidase (Amersham-Pharmacia) and developed with a chemiluminescence western blotting detection kit. The results indicate that the proprotein convertases required for correct processing of proinsulin to mature insulin (PC1/3 and PC2; Steiner, D.F., *Curr. Opin. Chem. Biol.* **2**:31 (1998)) were expressed in GTC-1 cells (FIG. 4, Bottom).

To confirm that proinsulin was appropriately processed, insulin and C-peptide levels in the cell culture media were measured (FIG. 5). Both C-peptide and insulin were detected in culture media collected from GTC-1 cell transfected with the GIP/Ins plasmid. This result indicates that K cells are process proinsulin to mature insulin.

To confirm that production of human insulin from GTC-1 cells transfected with the GIP/Ins plasmid was glucose regulatable, insulin levels in the cell culture media under different concentrations of glucose were assayed. In brief, 70-80% confluent GTC-1 cells in 12-well plates were fasted 2 hr in DMEM with 1.0 mM glucose and 1% Fetal calf serum (FCS). Cells were washed and then incubated in 0.5 mL of release media (DMEM plus 1% FCS with either 1.0 or 10.0 mM of glucose) for 2 hr. Medium was collected after 2 hours for each condition and assayed using the human-specific insulin ELISA kit according to the supplier's instructions (ALPCO). Furthermore, release of insulin from these cells was glucose-dependent (FIG 6).

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EXAMPLE II

This example describes transgenic mice that produce insulin in response to glucose.

Using the human insulin expression construct GIP/Ins described in Example I, the
5   GIP/insulin fragment (~4.1 Kb) was removed by digestion with HindIII. Transgenic mice were generated by pronuclear microinjection of the ~4.1 Kb transgene into fertilized embryos that were implanted into pseudopregnant females. Transgenic offspring were identified by Southern blot analysis. DNA from ear sections was digested with Xhol and PvuII (FIG 3), electrophoretically separated, and transferred to nylon membrane. For the
10   detection of the transgene, a 416 bp human insulin gene fragment encompassing intron 2 was amplified using primers 2 and 4 (FIG 3). The PCR product was prepared as a probe by random labeling with $[\alpha^{-32}P]$ dCTP, and bands were detected by autoradiography. Southern analysis results were further confirmed by PCR amplification of the genomic DNA using primers 2 and 4. Positive founders were outbred with wild-type FVB/N mice
15   to establish transgenic lines (FIG 8).

Transgenic mice tissues were examined for insulin expression. In brief, total RNA (50 μg) for each mouse stomach and duodenum, ileum, muscle, liver, spleen, kidney, fat, brain, lung, heart, bladder and testes were fractionated, transferred to a membrane and probed with a 333 base pair cDNA fragment encompassing exons 1 and 2 and part of exon
20   3 of human preproinsulin gene. The analysis revealed that insulin was expressed in the stomach and duodenum, but not in ileum, muscle, liver, spleen, kidney fat, brain, lung, heart, bladder or testes from the resulting transgenic animals (FIG 9).

To confirm insulin production in duodenum, RT-PCR analysis for insulin mRNA was performed. In brief, human proinsulin specific, forward 5'-
25   CCAGCCGCAGCCTTTGTGA-3' and reverse 5'-GGTACAGCATTGTTCCACAATG-3'; mouse proinsulin specific, forward 5'-ACCACCAGCCCTAAGTGAT-3' and reverse 5'-CTAGTTGCAGTAGTTCTCCAGC-3' primers used were. PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min for 45 cycles. PCR products were analyzed on a 2% agarose gel and visualized

by ethidium bromide staining. The human- and mouse- specific primer sets yield 350 bp and 396 bp products, respectively.

Insulin RNA was detected in the duodenum sample from the transgenic mice confirming that insulin was not due to contamination from adjacent mouse pancreas (FIG 9). Cellular localization of insulin protein was determined in tissue biopsies from transgenic mice utilizing antibody to insulin. Insulin immunoreactivity was detected in distinct endocrine cells in sections from stomach of transgenic animals (FIG 10).

The aforementioned results indicate that tissue distribution of insulin expression in transgenic animals corresponds to the tissue expression pattern of GIP (Tseng *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:1992 (1993); Yeung *et al.*, *Mol. Cell. Endocrinol.* **154**:161 (1999)).

To determine whether the cells that expressed insulin were K cells, tissues were analyzed for immune-reactivity with GIP antisera. In brief, tissues were fixed in Bouin's solution overnight and embedded in paraffin. Tissue sections (5 μm thick) were mounted on glass slides. For immunohistochemistry, the avidin-biotin complex method was used with peroxidase and diaminobenzidine as the chromogen. Sections were incubated with guinea pig anti-insulin (1:500; Linco Research, Inc.) or mouse anti-GIP (1:200; R. Pederson, University of British Columbia) for 30 min and appropriate secondary antibodies for 20 min at room temperature. Biotinylated secondary antibodies were used for immunohistochemistry, and fluorescein- or Cy3-conjugated secondary antibodies were used for immunofluorescence. The results indicate that insulin-expressing cells were K cells due to co-expression of immunoreactive GIP (FIG 10). These results confirm that human insulin production was effectively targeted to K cells in the gut of mice.

EXAMPLE III

This example shows that insulin production in transgenic mice provided normal glucose homeostasis and protection from developing diabetes. Production of human insulin from gut K cells of transgenic mice is also meal regulated. This example also describes data showing that glucose inducible insulin production by the transgenic mice provides glucose homeostasis after destruction of pancreatic β cells.

Analysis of plasma human insulin levels in transgenic mice in response to food intake was performed. In brief, plasma insulin levels were measured using the human-specific insulin ELISA kit (ALPCO) according to supplier's instructions. This assay has <0.01% cross-reactivity with human proinsulin and C-peptide and does not detect mouse

5   insulin. Plasma C-peptide measurements were made with a rat/mouse C-peptide RIA kit (Linco). The assay displays no cross-reactivity with human C-peptide.

In pooled plasma samples collected after oral glucose challenge, insulin was 39.0 ± 9.8 pM (n=10, Mean±SEM) in transgenic and undetectable in controls (n=5). To confirm that human insulin produced from K cells is meal regulated, transgenic mice were

10   fasted. Following a 40 hour fast, blood samples were collected via the tail vein. Animals were then refed with a standard chow and blood samples were collected again 24 hr after food replacement.

As shown in FIG. 11A, fasting significantly reduced the circulating human insulin in transgenic mice by more 40% (13.0 ± 4.2 pM vs 7.6 ± 2.3 pM, $p<0.03$). After food

15   restriction, refeeding resulted in over 400% increase in circulating human insulin.

To evaluate the release kinetics of human insulin from gut K cells, fasted transgenic mice were fed either a mixed meal in the form of a chow pellet (0.5g) or an oral glucose challenge (3 mg/g body weight). As shown in FIG. 11B, both oral nutrient challenges promptly stimulated the release of human insulin from gut K cells by at least

20   20% within 30 min. These results confirmed that insulin secretion from gut K cells is indeed meal-regulated.

Interestingly, levels of mouse C-peptide after an oral glucose load in transgenics were ~30% lower than controls (227.1 ± 31.5 pM vs 361.5 ± 31.2 pM, n=3 in each group, mean±SEM). This observation suggests that human insulin produced from the gut may

25   have led to compensatory down-regulation of endogenous insulin production.

The ability of human insulin production from gut K cells to protect transgenic mice from diabetes was investigated. Streptozotocin (STZ), a β-cell toxin, was administered to transgenic mice and age-matched controls. In brief, Streptozotocin (200 mg/kg body weight) in citrate buffer was administered to 8 week old transgenic and age-matched

control mice via an intraperitoneal injection. At this dose of streptozotocin, mice typically display glucosuria within 3 days post injection.

In control animals, STZ treatment resulted in fasting hyperglycemia (26.2 ± 1.52 mM, n=3, mean$\pm$SEM) and the presence of glucose in the urine within 3 to 4 days,

5 indicating the development of diabetes. When left untreated these animals deteriorated rapidly and died within 7 to 10 days. In contrast, neither glucosuria nor fasting hyperglycemia (9.52 ± 0.67 mM, n=5, mean$\pm$SEM) was detected in transgenic mice for up to three months after STZ treatment and they continued to gain weight normally.

To determine if insulin production from K cells was able to maintain oral glucose

10 tolerance in these mice despite the severe β-cell damage by STZ, mice were challenged with an oral glucose load five days after STZ treatment. In brief, glucose was administered orally by feeding tube (1.5 g/kg body weight) as a 40% solution (wt/vol) to mice fasted for 14 hr. Blood samples (40 μl) were collected from the tail vein of conscious mice at 0, 10, 20, 30, 60, 90, and 120 minutes following the glucose load.

15 Plasma glucose levels were determined by enzymatic, colorimetric assay (Sigma) and plasma insulin levels were measured using human-specific insulin ELISA kit (ALPCO).

Control mice given STZ were severely hyperglycemic both before and after the glucose ingestion (FIG 12). In contrast, STZ-treated transgenic mice had normal blood glucose levels and rapidly disposed of the oral glucose load as did normal age-matched

20 control mice (FIG 12).

To ensure that the STZ treatment effectively destroyed the β-cells in these experimental animals, pancreatic sections from controls and STZ-treated transgenic animals were immunostained for mouse insulin as previously described. The number of cell clusters positively stained for mouse insulin was substantially lower in STZ-treated

25 animals when compared to sham-treated controls (FIG 13). Total pancreatic content of insulin in STZ-treated transgenic mice was assessed by homogenizing pancreata and sonication at 4°C in 2 mM acetic acid containing 0.25% BSA. After incubation for 2 hr on ice, tissue homogenates were resonicated, centrifuged (8,000 g, 20 min) and supernatants were assayed for insulin by radioimmunoassayonly. The results indicate that total

30 pancreatic content in STZ-treated transgenic mice was 0.5% that of the sham-treated

controls (0.18 vs 34.0 µg insulin per pancreas, n=2). The fact that these STZ-treated transgenic mice disposed of oral glucose like normal mice despite having virtually no pancreatic β-cells indicates that human insulin produced in the gut was sufficient to maintain normal glucose tolerance.

5   These findings indicate that insulin production from gut K cells can protect the mice from developing diabetes and also provide normal glucose homeostasis to the extent of restoring normal glucose tolerance. Therefore, insulin expressed in gut is a means with which hyperglycemic conditions such as diabetes can be treated.

EXAMPLE IV

10   This example describes transplanting a transformed cell that produces a protein in response to nutrient into a tissue of a mammalian subject.

To isolate target mucosal cells, a tissue biopsy will be collected from the duodenum of a subject. The biopsy is washed in ice-cold Hanks' balanced-salt solution (HBSS; Gibco BRL) ~pH 7.4 containing 0.1% bovine serum albumin (BSA; Sigma) and 15   finely chopped with scalpels followed by digestion in an enzyme mixture containing 75 U/ml type I collagenase (Sigma), 75 U/ml type XI collagenase (Sigma), 0.9 U/ml type IX collagenase (Sigma) and 1 U/ml trypsin (Worthington Biochemical Corp) for 1 hour in a shaking water bath at 37°C. The total volume is then doubled with HBSS-BSA and allowed to settle for 10 min. The supernatant containing detached cells is discarded. The 20   remaining tissue is further digested in the enzyme mixture for two 45 min periods, with each step followed by the addition of 300 µl of 0.5 M EDTA for 15 min. The cell suspension resulting from digest 3 is filtered through Nitex mesh (200 µm, B&SH Thompson) and washed and centrifuged at 200xg twice with HBSS-BSA supplemented with 0.01% dithiothreitol and 0.001% DNase. The cells are then filtered a second time 25   through fine Nitex mesh (62 µm, B&SH Thompson), counted, and diluted in HBSS-BSA-DTT-DNase to 6×10^6 cells/ml for elutriation.

Mucosal endocrine cells will be enriched using a counter-flow centrifugal elutriation of cells (Lindahl P.E. Nature 161:648 (1948), a procedure that separates cells on the basis of their sedimentation coefficients. The cell suspension is pumped into a

rotating chamber, and cells are held where their sedimentation rate is balanced by the flow of fluid through the separation chamber. Different fractions of homogeneous cells are then 'eluted' by either increasing the flow rate through the chamber or decreasing the centrifugal speed. The appropriate flow rates and centrifugation speeds are determined

5   empirically. Batches consisting of 1.5 x 10^8 dispersed cells are introduced into the Beckman elutriator (model J2-21 M/E; Beckman) via a pump (Cole Palmer) connected to a sterile source of HBSS-BSA.

The enzyme dispersed mucosal cells are loaded into the elutriator chamber at a rotor speed of 2500 rpm with a flow rate of 25 ml/min and washed for 2 min. A 100 ml

10   fraction (F1) is collected after changing the flow rate to 30 ml/min. A second 100 ml fraction (F2) is obtained at a rotor speed of 2100 rpm and a flow rate of 55 ml/min. Cells from F2 are concentrated by centrifuging at 200 x g for 10 min, and then resuspended in sterile culture medium (DMEM (47.5%) and Ham's F-12K containing 5.5 mM glucose, 5% fetal calf serum, 2 ng/ml nerve growth factor, 8 mg/L insulin, mg/L hydrocortisone, 50

15   mg/L gentamycin, 0.25 mg/L amphotericin B, 50 U/ml penicillin, 50 mg/L streptomycin and 20 μM cytosineβ-D-arabinofuranoside.

The resulting mucosal endocrine cells are conditionally immortalized according to Kobayashi *et. al.* (Science **287**:1258 (2000)). Cultured mucosal cells are transduced with a standard replication incompetent retroviral vector harboring a genetic construct

20   consisting of the GIP promoter operably-linked to an oncogene (*e.g.* telomerase, large-T antigen, v-myc, ras, among others) tandemly fused to an IRES and a HSV-*tk gene*. The insulin and selection marker is expressed bi-cistronically. The genetic construct is flanked by recombinase recognition sites to allow for excision of the oncogene and hence deimmortalization of cells prior to transplantation.

25   To establish an immortalized K cell line, surviving clones of cells - transduced with the retroviral vector carrying the GIP promoter linked to an oncogene - are examined for the expression of GIP by immuno-fluorescence staining and western blotting. Clones that express satisfactory amounts of GIP are further expanded to established a K cell line. The K cell line is further transduced with a retroviral vector carrying a genetic construct

30   consisting of the GIP promoter operably linked to a nucleic acid encoding human insulin

and a positive selection marker. The insulin and selection marker is expressed bi-cistronically. The transfected K cells are incubated with appropriate selection drug. Surviving clones are isolated and tested for the expression of human insulin by western blot and ELISA (ALPCO).

5   K cell clone expressing appropriate levels of human insulin are cultured until sufficient number of cells are obtained. Prior to transplantation into a mammalian subject, the human insulin expressing K cell line is deimmortalized by excision of the oncogene. This is accomplished by transfecting cells with adenovirus expressing the appropriate recombinase (e.g. cre, flp, among others). Twenty-four to forty-eight hour after their
10   transfection, cells are incubated in gancyclovir. After 78 hrs exposure to gancyclovir, surviving cells (10^6-10^{12} cells) are purified and prepared for transplantation into a mammalian subject.

As an alternative, mucosal precursor cells or stem cells are isolated from duodenal biopsies by enzymatic (e.g. thermolysin) dissociation and expanded in culture as described
15   previously (Perraeault N. & Beaulieu J.F. *Exp Cell Res* **245**:34 (1998); Perraeault N. & Beaulieu J.F. *Exp Cell Res* **224**:354 (1996)). These stem cells and precursor cells are transfected with viral vectors carrying the GIP/Ins construct. Cells that are transfected successfully are selected by incubation in selection drug. These genetically engineered cells are then induced to differentiate and finally transplanted into mammalian subjects.

20   In summary, this example illustrates an *ex vivo* method for engineering K cells and mucosal endocrine precursor cells to produce human insulin. The engineered cells can be transplanted back into the same subject or to a different subject. The transplantation can be accomplished by several well established methodologies as disclosed herein or known in the art. For example, cells can be encapsulated and implanted under the skin of the
25   mammalian subject or cells can be implanted in the liver through portal delivery.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.